Mitochondria Distinguish Granule-Stored from de novo Synthesized Tumor Necrosis Factor Secretion in Human Mast Cells

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Key Words

Lipopolysaccharide • Mast cells • Mitochondria • Substance P • Tumor necrosis factor

Abstract

Background: Mast cells are immune cells derived from hematopoietic precursors that mature in the tissue microenvironment. Mast cells are critical for allergic, immune and inflammatory processes, many of which involve tumor necrosis factor (TNF). These cells uniquely store TNF in their secretory granules. Upon stimulation, mast cells rapidly (30 min) secrete β -hexosaminidase and granule-stored TNF through degranulation, but also increase TNF mRNA and release de novo synthesized TNF 24 h later. The regulation of these two distinct pathways is poorly understood. **Methods:** Human LAD2 leukemic mast cells are stimulated by substance P. TNF secretion and gene expression were measured by ELISA and real-time PCR, and mitochondrial dynamics was observed in live cells under confocal microscopy. Cell energy consumption was measured in terms of oxygen con-

sumption rate. **Results:** Here, we show that granule-stored TNF is preformed, and its secretion from LAD2 mast cells stimulated by substance P (1) exhibits higher energy consumption and is inhibited by the mitochondrial ATP pump blocker oligomycin, (2) shows rapid increase in intracellular calcium levels, and (3) exhibits reversible mitochondrial translocation, from a perinuclear distribution to the cell surface, as compared to de novo synthesized TNF release induced by lipopolysaccharide. This mitochondrial translocation is confirmed using primary human umbilical cord blood-derived mast cells stimulated by an allergic trigger (IgE/streptavidin). **Conclusion:** Our findings indicate that unique mitochondrial functions distinguish granule-stored from newly synthesized TNF release from human mast cells, thus permitting the versatile involvement of mast cells in different biological processes.

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Introduction

Mast cells are bone marrow-derived immune cells that can secrete prestored mediators such as histamine and tryptase through rapid (5–30 min) degranulation as well as delayed (12–24 h) newly synthesized cytokines including interleukin-4 (IL-4) IL-6, IL-8, IL-13 and tumor necrosis factor (TNF) in response to allergic or neuropeptide triggers [1, 2]. In fact, mast cells uniquely store TNF in secretory granules [3, 4]. Stimulation of LAD2 [5] cells by substance P (SP) induces degranulation and secretion of prestored TNF [6], while stimulation with lipopolysaccharide (LPS) induces selective de novo synthesis and release of TNF without degranulation [7–9].

Other secretory cell types, like eosinophils, use distinct mechanisms for secretion, such as exocytosis of large storage granules and release from small secretory vesicles [10]. Mast cells can also release mediators selectively without degranulation [11], first reported for the release of serotonin without histamine [12] and later for IL-6 without histamine [13]. In both cases, this involved release from small vesicles (80 nm diameter) rather than from the typical secretory granules (1,000 nm diameter) [13, 14]. This ability, i.e. mast cell stimulation by allergic and non-immune triggers [15], as well as the synergistic stimulation by cytokines and neuropeptides [16] may allow mast cells to participate in a variety of distinct pathophysiological settings, in addition to allergy [17]. These include innate and acquired immunity [18], inflammation [11], autoimmunity [19], wound healing [20] and cancer growth [21], as well as atherosclerosis and obesity [22]. However, little is known about what distinguishes rapid degranulation from delayed selective cytokine release.

Degranulation from rat peritoneal mast cells requires metabolic energy and calcium [23]. Mitochondria are the primary source of energy production in eukaryotic cells and also have the ability to buffer calcium locally [24]. Moreover, mitochondria are dynamic organelles that participate in many complicated cell functions through morphological and localization changes [25]. Increasing evidence indicates the importance of mitochondrial dynamics in immune cell regulation. For instance, local ATP production by mitochondria is required for T-cell chemotaxis [26]. Moreover, mitochondrial translocation is required for T-cell 'immune synapse' formation and sustainable calcium influx [27]. On the other hand, local intracellular calcium changes can regulate mitochondrial dynamics and subcellular localization [28].

In this study, we show that SP-induced granule-stored TNF secretion, unlike newly synthesized selective TNF release, requires high mitochondrial energy consumption, intracellular calcium increase and mitochondrial translocation to the cell surface.

Materials and Methods

Cell Lines and Reagents

LAD2 culture cells [5] (from Dr. A.S. Kirshenbaum, NIH, Bethesda, Md., USA) were cultured in StemPro-34 medium (Invitrogen, Carlsbad, Calif., USA) supplemented with 100 ng/ml recombinant human stem cell factor (Swedish Orphan Biovitrum Sverige AB, Stockholm, Sweden) and 100 U/ml penicillin/streptomycin. Cells were grown in an incubator in 5% CO₂ at 37°C. All cells were used during their logarithmic growth period. They were stimulated by either SP (10 µM) or LPS (10 ng/ml) dissolved in distilled water.

Human cord blood-derived mast cells (hCBMCs) were grown from human cord blood obtained during normal deliveries in accordance with established institutional guidelines [29]. Briefly, mononuclear cells were isolated by layering heparin-treated cord blood onto lymphocyte separation medium (ICN Biomedical, Aurora, Ohio, USA). CD34+ progenitor cells were isolated from mononuclear cells by positive selection of AC133 (CD133+/CD34+) cells by magnetic cell sorting (Miltenyi Biotec, Auburn, Calif., USA). hCBMCs were derived by the culture of CD34+ progenitor cells with minor modifications. For the first 6 weeks, CD34+ cells were cultured in AIM medium (Gibco, Grand Island, N.Y., USA) supplemented with 100 ng/ml recombinant human stem cell factor, and after 6 weeks, 50 ng/ml IL-6 (Chemicon, Billerica, Mass., USA) was added and cultured at 37°C in 5% CO₂ balanced air. Mast cell viability was determined by trypan blue (0.3%) exclusion. Cells were stimulated first, passively sensitized with human monoclonal IgE (1 µg/ml; EMD Bioscience, Darmstadt, Germany) for 24 h and then with streptavidin (125 ng/ml; Sigma, St. Louis, Mo., USA) for 30 min as indicated [30].

TNF Secretion Assay

LAD2 cells were treated with SP (10 µM; Sigma), in order to achieve the strongest possible degranulation, or with LPS (10 ng/ml; Sigma) for 30 min, 6 and 24 h. TNF release was measured by ELISA (R&D Systems, Minneapolis, Minn., USA) in the supernatant fluid. In certain experiments, LAD2 cells were pretreated with the transcription inhibitor actinomycin D (15 µM for 1 h; Sigma) before stimulation with SP.

Mast Cell Degranulation Assay

β-Hexosaminidase (β-hex) secretion, as an index of mast cell degranulation, was assayed using a fluorometric assay as previously reported. Briefly, β-hex activity in the supernatant fluid and cell lysates (LAD2 cells, 0.5 × 10⁵/tube, were lysed with 1% Triton X-100 to measure residual cell-associated β-hex) were incubated with substrate solution (*p*-nitrophenyl-N-acetyl-β-D-glucosaminide; Sigma) in 0.1 M NaOH/0.2 M glycine. Absorbance was measured at 405 nm in an enzyme-linked immunosorbent assay reader, and the results are expressed as the percentage of β-hex released over the total.

Fig. 1. Time course of SP and LPS-induced TNF secretion, mRNA expression and light microscopy of LAD2 cells. LAD2 cells were treated with SP (10 μ M) or LPS (10 ng/ml) for the time indicated. * $p < 0.05$; ** $p < 0.01$. **a** TNF release was measured by ELISA (R&D Systems) in the supernatant fluid ($n = 3$). **b** TNF mRNA expression was measured by real-time PCR ($n = 3$). **c, d** LAD2 cells were stained with toluidine blue and observed under light microscopy. LAD2 cells were treated with SP (10 μ M) for 30 min (**c**) and 24 h (**d**). Scale bars = 5 μ m. **e** β -hex release was measured ($n = 3$). **f** TNF release was measured from cells treated with SP with or without actinomycin D (ActD) preincubation (15 μ g/ml for 60 min; $n = 3$).

Quantitative Reverse Transcription PCR

Total RNA from cultured mast cells was isolated using Trizol reagent (Invitrogen) and RNeasy Mini Kit (Qiagen, Valencia, Calif., USA), respectively, according to the manufacturer's instructions. Reverse transcription was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, Calif., USA). In order to measure TNF gene expression, quantitative real-time PCR was performed using Taqman gene expression assays. The following probes (Applied Biosystems, Carlsbad, Calif., USA) were used: TNF: Hs00542477_m1. Samples were run at 30 cycles using Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems). Relative mRNA abundance was determined from standard curves run with each experiment, and TNF expressions were normalized to GAPDH (Hu, VIC TAMRA) as endogenous control.

Intracellular Calcium Measurement

LAD2 cells were loaded with 5 μ M Fura-2 AM (Invitrogen) for 20 min, washed and incubated for another 20 min at 37°C. Cells were then treated with either SP (10 μ M) or LPS (10 ng/ml). Fluorescence signals were acquired on Flexstation II (Bücher Biotech, Basel, Switzerland).

Cytosolic calcium was calculated after subtraction of the background fluorescence by measuring the ratio of the two emission intensities (excitation at 340 and 380 nm). Each experiment was repeated three times independently.

Cell Energy Consumption Measurement

LAD2 cell oxygen consumption rates were measured by Seahorse XF-24 Flux analyzer (Seahorse Bioscience Inc., North Billerica, Mass., USA). LAD2 cells were treated with SP (10 μ M) or LPS (10 ng/ml). Energy consumption was inhibited by the mitochondrial ATP pump blocker oligomycin. Cells were incubated with oligomycin (2 μ M for 20 min; Sigma) and then treated with SP (10 μ M; Sigma) or LPS (10 ng/ml; Sigma). Experiments were conducted three times and results were similar.

Confocal Microscopy

Human mast cells were incubated with 20 nM MitoTracker deep red probe (Invitrogen) for 20 min and 50 nM LysoTracker DND (Invitrogen) for 30 min. Cells were washed, moved to glass

Fig. 2. Time course of SP and LPS stimulation on LAD2 mast cell cytosolic calcium changes. LAD2 cells were loaded with 5 μM Fura-2 AM (Invitrogen) for 20 min at 37°C, washed and incubated for another 20 min. Cells were then treated with either SP (10 μM) or LPS (10 ng/ml). Fluorescence signals were acquired on Flexstation II. The figure is representative of three repeats with similar results.

bottom culture dishes (MatTek, Ashland, Mass., USA) and observed using a Leica TCS SP2 confocal microscopy (Leica, Buffalo Grove, Ill., USA). Percentages of cells with mitochondrial translocation were counted from 100 randomly selected mast cells in each experiment by three independent operators. Confocal digital images were processed using the National Institute of Health ImageJ 1.32 and Adobe Photoshop 7.0 programs.

Data Analysis

Statistically significance differences between experimental samples and controls were determined by the Student *t* test using SigmaPlot 9.0 (SPSS, Chicago, Ill., USA). Differences were considered significant if $p < 0.05$.

Results

Time Course of SP and LPS-Induced TNF Secretion and Gene Expression

SP stimulation (10 μM) results in secretion of granule-stored TNF at 30 min (fig. 1a) as well as of de novo synthesized TNF 24 h later (fig. 1a). Stimulation with LPS (10 ng/ml) for 30 min has no effect on degranulation and granule-stored TNF (fig. 1a), but incubation for 24 h induces de novo synthesis and release of TNF without degranulation (fig. 1a). The gene expression level of TNF is significantly increased at 6 and 24 h both after SP and LPS stimulation (fig. 1b), indicating that SP and LPS induce de novo TNF synthesis. Under light microscopy, LAD2 cells stimulated by SP at 30 min show clear signs of degranulation (fig. 1c), but no signs of degranulation at

24 h (fig. 1d). β -hex is also measured to confirm degranulation, and secretion occurred in parallel with preformed TNF (fig. 1e). In order to confirm that TNF secreted at 30 min is preformed, LAD2 cells are treated with RNA synthesis blocker actinomycin D (15 $\mu\text{g/ml}$) 1 h before SP stimulation. There is no significant difference in TNF release amounts at 30 min with or without actinomycin D (15 $\mu\text{g/ml}$) (fig. 1f).

Intracellular Calcium Levels of SP and LPS-Induced TNF Secretion

SP (10 μM) triggers a rapid significant cytosolic calcium increase within 1 min (fig. 2a). SP resulting in cytosolic calcium level returns to the same level of control after 50 min. LPS (10 ng/ml) has no effect on intracellular level (fig. 2b).

Energy Consumption during SP-Stimulated Preformed and LPS-Induced de novo Synthesized TNF Release

To test if degranulation and de novo TNF secretion have different energy requirements, mitochondrial oxygen consumption was investigated during these two processes in LAD2 cells. SP induced a significant oxygen consumption spike, while there was almost no difference between LPS-stimulated and control cells (fig. 3a). In order to investigate if mitochondrial energy production is required for degranulation, mitochondrial energy production was blocked by pretreating LAD2 cells with the

Fig. 3. Energy consumption in LAD2 cells stimulated by SP and LPS. **a, b** Cell oxygen consumption rates were measured by Seahorse XF-24 Flux analyzer (Seahorse Bioscience Inc.) in LAD2 cells treated with either SP (10 μ M) or LPS (10 ng/ml). LAD2 cells were pretreated (**b**) by the mitochondrial ATP pump blocker oli-

gomyacin (2 μ M) for 20 min and were then treated with SP (10 μ M) or LPS (10 ng/ml) for 30 min or 24 h, respectively. Experiments were conducted three times and one representative experiment is shown. TNF secretion was measured by ELISA as indicated before (n = 3). Spon = Spontaneous. * p < 0.05.

ATP synthase inhibitor oligomycin (2 μ M) for 30 min. Oligomycin treatment dropped the metabolic baseline of both SP- and LPS-treated cells to 30% of normal. In addition, the energy consumption spike (fig. 3b) was inhibited. Oligomycin treatment also inhibits SP-stimulated granule-stored TNF secretion at 30 min (fig. 3c). In contrast, there is no significant difference in SP-induced TNF release at 24 h with or without treatment with oligomycin (fig. 3d).

Degranulation, but Not de novo Cytokine Secretion, Results in Mitochondrial Translocation

Examination of resting live LAD2 cells by confocal microscopy shows that mitochondria stained with Mito-

Tracker red are located around the nucleus as a 'mitochondrial pool' (indicated by the white dashed circle); very few mitochondria could be found close to the cell surface (fig. 4a). Since the average pH of mast cell granules is 5.5 [31], the lysosome dye LysoTracker [32] was used to stain secretory granules. After SP (10 μ M) stimulation for 30 min at 37°C, mast cells undergo rapid degranulation as indicated by the content of numerous granules stained with LysoTracker outside the cell (fig. 4b, left panel). Following degranulation, many mitochondria appear to be smaller (fig. 4b, middle panel) and to have translocated close to the cell surface region (fig. 4b, right panel). This phenomenon is not observed in LPS-stimulated mast cells (fig. 4c).

Fig. 4. Degranulation, but not de novo TNF release, is associated with mitochondrial translocation. LAD2 cells were stained with MitoTracker deep red probe (20 nM) for 20 min and LysoTracker DND green probe (50 nM) for 30 min. The cells were harvested in glass bottom culture dishes and observed under the Leica TCS SP2 confocal microscope. The left panels depict granules in green and the middle panels represent mitochondrial fluorescence shown in red. The right panels represent images merged from the

two previous panels. The white dashed circles outline the 'mitochondrial pool'. The graphs shown at the far right represent percentages of mast cells with mitochondrial translocation obtained from 100 randomly selected cells. **a** Control groups. **b** SP (10 μ M, 30 min). **c** LPS (10 ng/ml, 30 min). Each experiment was repeated three times and was evaluated by three independent operators. Scale bars = 5 μ m. The colors refer to the online version of the figure.

Degranulation-Associated Mitochondrial Translocation Is Reversible

Confocal images of SP-activated mast cells at 24 h show that there is no evidence of degranulation (fig. 5b). At 24 h, it is obvious that most mitochondria were found again in the perinuclear region (fig. 5b). Only about 20% of cells contain translocated mitochondria, which is not different from that of controls. This finding indicates that degranulation-induced mitochondrial translocation is reversible. Just as there is no mitochondrial translocation 30 min after LPS stimulation (fig. 4c), there is no translocation at 24 h either; LPS-stimulated mast cells still con-

tained intact mitochondria located in the perinuclear region (fig. 5c).

IgE/Streptavidin-Stimulated hCBMCs Secrete Preformed TNF, Associated with Mitochondrial Translocation

Under confocal microscopy, unstimulated hCBMCs (fig. 6a, upper panels), as well as those treated only with IgE (1 μ g/ml) for 30 min (fig. 6a, middle panels), had most of their mitochondria interconnected in a 'net' located in the perinuclear region (within the dashed circles). In contrast, hCBMCs stimulated by IgE (1 μ g/ml)

Fig. 5. Degranulation-associated mitochondrial translocation is reversible. LAD2 cells were stained with MitoTracker deep red probe (20 nM) for 20 min and LysoTracker DND green probe (50 nM) for 30 min. The left panels depict granules in green and the middle panels represent mitochondrial fluorescence. The right panels represent images merged from the two previous panels. The white dashed circles outline the 'mitochondrial pool'. The

graphs at the far right represent percentages of mast cells with mitochondrial translocation from 100 randomly selected cells. **a** Control groups. **b** SP (10 μ M, 24 h). **c** LPS (10 ng/ml, 24 h). Each experiment was repeated three times and was calculated by three independent operators. Scale bars = 5 μ m. The colors refer to the online version of the figure.

and streptavidin (125 ng/ml) show rapid (30 min) degranulation (fig. 6c) at up to 35% β -hex release (fig. 6d) and mitochondrial translocation (fig. 6c, lower panels).

Discussion

The present findings show that TNF secretion from human mast cells can occur through distinct pathways with different energy and calcium requirements, as well as mitochondrial dynamics. Degranulation is known to require calcium [23] and energy [33]. Here, we show that

this process requires much more energy and calcium than de novo synthesized TNF release. The preformed nature of rapid released TNF was confirmed by the treatment of transcriptional inhibitor actinomycin D which has little effect on blocking rapid TNF release. We used a high SP concentration (10 μ M) in order to induce maximum degranulation. Such a high concentration has also been used previously to induce maximal LAD2 cell activation [6]. Moreover, only SP concentrations >1 μ M were reported to be able to enhance the rate of oxygen consumption of isolated cardiac cell mitochondria [34]. Other authors have reported secretion of preformed TNF

from mast cells in different species by different triggers [4, 35, 36]. However, there was no attempt to investigate how this secretion differs from de novo synthesis and release. Delayed (24 h) TNF release could not be from continuous degranulation because morphological observations of SP-stimulated mast cells showed no degranulation at 24 h. It would have been desirable, instead of SP, to use peptidoglycan, which has been reported to induce degranulation from rodent mast cells [37]. However, LAD2 cells are unresponsive to peptidoglycan [38].

We especially show that mast cell-preformed TNF secretion is associated with mitochondrial translocation to the cell surface. Mitochondrial translocation was confirmed using hCBMCs stimulated by IgE/streptavidin. These results indicate that such mitochondrial translocation is not due to any specific trigger or the leukemic nature of LAD2 cells. We recently showed that mast cell degranulation is tightly associated with mitochondrial morphological changes [39] and functions [40]. One possible explanation for our findings is that mitochondria

translocate close to the secretory granules in order to provide energy locally, possibly for the granules to fuse with the plasma membrane and undergo exocytosis as shown for lymphocyte chemotaxis [26]. Mitochondrial translocation may also be needed to maintain optimal local calcium levels necessary for exocytosis [23], most likely for the calcium-dependent proteins involved in degranulation, such as the soluble N-ethylmaleimide-sensitive factor attachment protein and the vesicle-associated membrane protein 8 [41]. It was previously shown that mitochondrial translocation was necessary to keep calcium channels open at the 'immunological synapse' in activated T cells [27].

Mitochondrial health is maintained through autophagy [42, 43]. The normal appearance and distribution of mitochondria in mast cells 24 h after SP stimulation may be either because fragmented mitochondria underwent fusion and translocated back to the perinuclear region or because there was biogenesis of new mitochondria and digestion of old mitochondria through autophagy. The latter possibility is supported by a recent paper reporting that inhibition of autophagy blocks mast cell secretion [44]. Unlike degranulation, selective mast cell release of de novo synthesized mediators [11] could involve vesicular traffic that requires negligible and hard to measure calcium and energy levels. For instance, differential release of serotonin without degranulation involved small vesicles shuttling serotonin from secretory granules to the cell surface [45]. Similarly, IL-1 induced selective release of de novo synthesized IL-6 contained within small vesicles [13]. Vesicular secretion may also be involved in the ability of corticotropin-releasing hormone to induce

selective release of vascular endothelial growth factor without degranulation [46].

The regulatory processes investigated here may be applicable to the release of the mediators and could help explain how human mast cells participate in diverse biological processes. Mitochondrial dynamics may be necessary for a rapid mast cell response to an environmental trigger, as opposed to a delayed mediator release in other conditions, such as inflammation [47], innate and acquired immunity [1] or metabolic diseases [22]. An implied importance of our findings could be the possibility of inhibition of mast cell mitochondrial translocation targeted as antiallergic therapy, while permitting de novo synthesis of molecules, such as vascular endothelial growth factor which is useful in wound healing. A better understanding of the mitochondrial dynamics involved in mast cell activation may permit individualized therapy for allergic and inflammatory disorders.

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